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Cancer Cells by Estrogen Regulation of Proteinase
Inhibitor 9

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13. ABSTRACT (Maximum 200 Words) Coagulation factor VIIa, a circulating serine protease, binds to its cofactor, tissue factor (TF), to trigger the blood clotting cascade. TF is a glycoprotein present on the surface of a variety of cell types found outside of the vasculature, and on various types of cancer cells. TF:VIIa complex formation ultimately leads to fibrin polymerization and platelet activation, in both normal states and thrombotic disease. Complex formation has also been shown to activate certain signaling cascades, altering cellular properties such as adhesion, migration, and potential for apoptosis. Many types of cancer cells have been shown to express high levels of TF. Blockage with anti-TF antibodies has demonstrated that metastasis depends on the presence of catalytically competent TF:VIIa complexes. Such cellular alterations, the high morbidity associated with thrombosis, and the privileged position of VIIa in the coagulation cascade, make inhibition of TF:VIIa an important issue in cancer therapy. We have demonstrated that antithrombin (AT) can inhibit TF:VIIa, when in the presence of heparin, and AT:VIIa complexes have been detected in plasma, suggesting that VIIa is inhibited by AT <i>in vivo</i> . We have also shown that vitronectin enhances AT activity, and hence, VIIa inhibition, and that AT can reversibly inhibit VIIa <i>in vitro</i> .				
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The Effects of Antithrombin and Tissue Factor Pathway Inhibitor on the Tissue Factor:Factor VIIa Complex on the Surface of Breast Cancer Cells: Implications on Blood Coagulation, Gene Expression, Cell Adhesion and Metastasis.

Annual Summary Report

Francesca Antonaci (Award Number DAMD17-02-1-0410)

Introduction: Coagulation factor VIIa (VIIa) is a circulating serine protease which, upon binding to its cofactor, tissue factor (TF), is responsible for triggering the blood clotting cascade in both normal hemostasis and in thrombotic disease (reviewed in 1). TF is a type I integral membrane protein present on the surface of a variety of cell types found outside of the vasculature, and on various types of cancer cells (2-5). The TF:VIIa complex catalyzes the maturation of factors IX and X to the activated factors IXa and Xa by limited proteolysis. Activation of these coagulation factors ultimately leads to fibrin polymerization and platelet activation, thereby plugging the vessel wall at the site of injury, or forming a clot within the vasculature, in the case of thrombotic disease and cancer. Binding of VIIa to TF has also been shown to activate certain signaling cascades, such as MAP kinase pathways, and to increase intracellular calcium levels, thus altering cellular properties such as adhesion, migration, and potential for apoptosis (reviewed in 6, 7). Many types of cancer cells have been shown to express high levels of tissue factor, and blockage of VIIa binding by use of anti-TF antibodies has demonstrated that metastasis, in these cells, is dependent on the presence of catalytically competent TF:VIIa complexes (8).

Alteration of cellular properties due to signaling through TF, the high morbidity associated with thrombosis in various disease states, and the privileged position of VIIa in the coagulation cascade, make inhibition of the TF:VIIa complex a very important issue which requires further study. There is currently only one human protein that is widely accepted as an inhibitor of the TF pathway, namely, tissue factor pathway inhibitor (TFPI) (reviewed in 9). TFPI, however, can only inhibit TF:VIIa after binding to an activated Xa molecule, and the active inhibitor is not found in high levels in plasma, leading to the notion that there may be more than one physiological inhibitor of the TF pathway. Some laboratories were able to show that antithrombin (AT), a serine protease inhibitor (serpin) that is most well known as an inhibitor of thrombin (factor II) and factor Xa, can also inhibit TF:VIIa, when in the presence of the glycosaminoglycan (GAG), heparin (10-12). The presence of AT:VIIa complexes has been demonstrated in plasma; the concentration of these complexes is higher than the concentration of free VIIa, demonstrating that VIIa can be inhibited by AT (unpublished lab results). I have recently demonstrated that the inhibition of TF:VIIa by AT/heparin has a rate constant of approximately $2.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. The rate constant for TFPI inhibition of TF:VIIa is much higher ($1.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) (13), but the plasma concentration of AT is $5 \mu\text{M}$, whereas active TFPI is present in very low levels in the vasculature under normal conditions (14), suggesting that AT may be at least as important as a physiological inhibitor of TF:VIIa as TFPI. The plasma half life of VIIa is also rather high, at approximately 5 hours, much higher than other coagulation factors which have half lives on the order of minutes. This would allow for an inhibitor that is much slower than traditional inhibitors, including

TFPI, because of the lack of time restraint. Although AT requires heparin for efficient inhibition of TF:VIIa, I postulate that glycosaminoglycans, such as heparan sulfate, present on the endothelium of vessel walls may be able to activate antithrombin similarly to heparin, thereby leading to inactivation of VIIa. It is also possible that there are other factors in plasma, not present in our *in vitro* assays, that may enhance the activity of AT, and allow for a more efficient inhibition of VIIa.

Results: During the past year, I have focused my efforts on gathering evidence to support the hypothesis that AT is, in fact, an important physiological inhibitor of VIIa; I have also tried to demonstrate that AT can reversibly inhibit VIIa in the absence of TF, and have studied whether this inhibition occurs by a competitive mechanism. I first spent time trying to develop and optimize an appropriate protocol for determining the rate constant for inhibition of TF:VIIa by AT/heparin. I then determined how the rate constant was affected by two different monoclonal antibodies against TF. I found that one antibody (5G9) binds so that X activation is completely blocked, and AT inhibition is greatly reduced. Most likely, 5G9 binding to TF causes steric hindrance that does not allow X or AT to bind, or to bind as tightly. Another antibody, 10H10, does not block X activation, but does partially block AT inhibition of TF:VIIa. These studies may be helpful in the future for mapping exosite interactions with AT, which would be useful in determining how AT may bind to and inhibit VIIa and TF. These may also serve useful in breast cancer cell studies because of their interactions with TF, VIIa, and potentially, AT.

I next concentrated my efforts on determining the effects of AT on VIIa activity in the absence of TF. I have repeatedly been able to show that AT can inhibit VIIa in the absence of TF, but that heparin is required to activate AT. I used a small chromogenic substrate to assay for VIIa activity, and found that 70% inhibition can be achieved, but that higher percentages of inhibition are not possible, indicating an equilibrium suggestive of a reversible reaction. Upon dilution of the above reaction, full recovery of VIIa activity is seen, confirming that this inhibition is indeed reversible in the absence of TF. Because results with a small substrate cannot fully mimic VIIa activity on X activation *in vivo*, I next tested the effects of dilution of AT reactions in clotting assays. Clotting assays were performed by adding pooled normal plasma to reactions with VIIa, AT, heparin, and TF. The reactions were pre-incubated, and then diluted and reincubated to allow for dissociation of antithrombin from VIIa. I found that clotting time is severely delayed when AT and VIIa are incubated in the presence of TF, and that dilution of these reactions does not allow for full recovery of activity. When TF is not present, AT is able to delay the clotting time, but after dilution, full recovery of activity can be achieved, further demonstrating that there may be an additional novel mechanism for AT inhibition of VIIa. I have used Western blotting to determine what levels of covalent complex formation is seen using various reaction conditions. Covalent complexes are identified as an up-shifted band visible after SDS-PAGE electrophoresis. High levels of these complexes are seen after incubation of VIIa with AT, heparin, and soluble TF. Very low levels of the complex are seen when TF is absent, indicating that most inhibition seen in *in vitro* assays is due to an interaction that does not result in covalent complex formation. Complex formation is very dependent on the presence of heparin, but GAGs on cell surfaces of the vessel walls may be able to substitute for heparin in activating AT. To

determine by which type of mechanism this inhibition is occurring, I began studies to determine V_{MAX} and K_M , but have thus far been unsuccessful in obtaining repeatable results. The chromogenic substrate used in these assays precipitates at higher concentrations, which has made it difficult to collect the amount of data that I need to determine V_{MAX} and K_M . I have identified a fluorogenic substrate that may be useful in these determinations, and these experiments are currently underway. Preliminary data suggests that the mechanism may be of a traditional competitive nature, that is, by way of an active site interaction, but more experiments will need to be completed in order to verify these results.

I have also begun work to probe for AT:VIIa complexes in plasma using a simple co-immunoprecipitation procedure. It is important to mention that our anti-VII antibody is successful at co-immunoprecipitating full length AT, as well as AT in complex with VIIa, providing further evidence that VIIa can interact with AT in a non-covalent manner for long periods of time. Complexes are seen in plasma alone, but are difficult to detect due to lack of sensitivity of the Western blotting procedure. When 100nM VIIa is added back to the plasma, large amounts of AT and AT:VIIa complexes precipitate with the antibody. Heparin and soluble TF enhance the precipitation several fold, but are not required for complex formation at these high concentrations. It would be of interest to add cells to the assay which express GAGs on their surfaces, but not TF, to determine whether they can enhance complex formation, as heparin does.

Finally, I have very preliminary data showing that vitronectin, a very abundant plasma protein, when added to assays to determine the rate constant for inhibition of VIIa by AT, can boost AT activity by as much as 6 fold in the absence of heparin, and 9 fold in the presence of heparin. This could be important because it may explain why AT does not seem to be a very important inhibitor *in vitro*, and show that it could be important in the context of plasma. It is also possible that there are other factors in plasma that could provide additional activation of AT. Co-immunoprecipitation studies followed by staining and sequencing may allow us to identify which proteins are bound to VIIa in plasma, and whether these proteins are able to increase AT's efficiency.

Conclusions and Future Directions: Although the rate constant for inhibition of TF:VIIa by AT/heparin is quite low, it still may be significant enough to play an important physiological role. TFPI levels are low in plasma, indicating that there may be another circulating inhibitor of VIIa. There are normally small amounts of active VIIa in blood, but the question then remains why blood clotting does not normally occur spontaneously, even though there have been reports of TF circulating in plasma (reviewed in 15). One must also wonder why cancer does not occur more often, and whether loss of regulation of VIIa activity in plasma can lead to development of cancerous states in cells in contact with plasma. We hypothesize that VIIa must circulate in a reversibly inhibited form, perhaps bound to antithrombin. Further studies must be completed on the type of inhibition mechanism AT uses in the absence of TF. It is also necessary to study the effects of endothelial and cancer cells with GAGs present on their surfaces on AT inhibition of VIIa activity in the presence and absence of TF; additional studies on the effects of vitronectin on AT activation are needed. The next step would be

to test various breast cancer cells to see what effects AT has on X activation and TF signaling by assaying for changes in cell adhesion, metastatic potential, apoptosis, and other parameters. I also hypothesize that AT inhibition of TF might be important in blocking the propagation of blood coagulation, and that, since the rate constant is so low, it might be in place in order to allow coagulation to initiate and propagate normally, but that it then acts as a brake to ensure that wide-spread coagulation does not occur. These hypotheses provide novel roles for AT in inhibition of blood proteases, and support the role of heparin as an important anticoagulant. This may pave the way for the use of these antithrombotics in cancer therapy.

Key Accomplishments:

- Determined rate constant for inhibition of VIIa:TF by AT in the presence of heparin;
- Demonstrated a reversible mechanism of inhibition of VIIa by AT in the absence of TF but in the presence of heparin;
- Showed that vitronectin, an important component of plasma, can enhance AT inhibition of VIIa:TF;
- Have made progress in determining the class of inhibition of VIIa by AT.

Problems: Since last year I have changed advisors. In the summer of 2002, I decided to leave Dr. Shapiro's lab to pursue my interests elsewhere. In my former lab I was not making progress in breast cancer, and was required to spend my time on other diseases such as cardiovascular disease and osteoporosis. After I left the lab I was required to do two 6-week rotations to decide on a new laboratory. I decided on the laboratory of Dr James H Morrissey, a well known TF researcher. I was asked to resubmit my proposal to reflect my new project, and the proposal was re-approved this spring. I am attaching a copy of the new proposal (see Appendix pages A-N), because as I understand the reviewers do not have access to internal files. This year I have concentrated on setting up my new project, and on preliminary studies with pure proteins to optimize assay conditions for future work with breast cancer cells. I was required to retake my preliminary examination after changing advisors, despite the fact that I had previously passed the exam. Just two weeks ago, I again passed the exam, but it took a lot of my time to prepare for it. In the past months I have also lost both of my grandmothers, and my father has been diagnosed with metastatic lung cancer. This has caused some loss of time in the laboratory for attendance at funerals, etc. I am, however, confident that I will make a lot of progress in the two years to come, and feel that I will do a good job at completing the project I outlined in my revised statement of work. All assay conditions have been optimized now, and I am ready to begin cell studies. I have not yet completed my first task, but as outlined in the statement, I will have finished it within the first 16 months of work; I have done important experiments *in vitro* in order to set up experiments *in vivo*.

Reportable Outcomes:

Presentations:

- Preliminary Examination, September 2003; passed with a recommendation of "outstanding;"

- Poster presentation at Medical Scholars Program fall retreat; title: Determining the Nature of Inhibition of VIIa by AT: A Possible Novel Mechanism for Maintaining Hemostasis (included on Appendix page O);

Conferences Attended:

- International Society of Thrombosis and Haemostasis Meeting in Birmingham, England, July 2003;
- Second International Conference on Thrombosis and Hemostasis Issues in Cancer, Bergamo, Italy, September 2003.

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The Effects of Antithrombin and Tissue Factor Pathway Inhibitor on the Tissue Factor:Factor VIIa Complex on the Surface of Breast Cancer Cells: Implications on Blood Coagulation, Gene Expression, Cell Adhesion and Metastasis

Proposal;

P.I. Francesca Antonaci

Background/Relevance

It has long been known that thrombosis, or the pathological formation of a blood clot within the vascular space, is of major importance in many types of cancer, including breast cancer. Since Trousseau's discovery in 1872 that thrombosis frequently complicates cancer, much attention has been given to the study of this phenomenon (1). In fact, approximately 10% of patients with deep vein thrombosis may also have an underlying carcinoma (2). Many studies have cited thrombosis as a major cause of complications in the treatment of breast cancer, one determining that 17.6% of metastatic breast cancer patients tested had also had thromboses (3). Many studies have shown that many cancer cells, including some breast carcinoma cells, express tissue factor (TF), the glycoprotein responsible for triggering the extrinsic blood coagulation cascade (4-10). Physiological hemostasis, or normal blood coagulation, occurs when a blood vessel incurs an injury, exposing TF present on the surface of the underlying cells. When TF contacts the blood, circulating factor VIIa binds tightly to it, forming a complex that can mature factors IX and X to their active enzymatic forms, factors IXa and Xa, by limited proteolysis. Alternatively, factor VII, the inert precursor to factor VIIa, can bind to TF and undergo an auto-activation reaction to form the TF:VIIa complex. These are the initiating events that lead to the formation of a blood clot, the necessary healthy outcome after blood vessel injury. However, since certain cancer cells, tumor-associated macrophages and endothelial cells also express TF and may be in contact with blood, especially in cases of metastasis, they can trigger the formation of a thrombus within the circulation, leading to events such as myocardial infarction, deep vein thrombosis (DVT) or stroke, pathological conditions with especially high mortality rates. Another complication is that certain chemotherapeutic drugs, such as cyclophosphamide, methotrexate, fluorouracil, vincristine, and prednisone, have been shown to precipitate thrombosis, despite lack of evidence in these patients of residual malignant disease (11). The seriousness of these conditions requires a better understanding of the events leading to thrombosis and how to prevent or treat them.

The TF:FVIIa complex plays a major role in blood coagulation, but it has recently been shown to have many other roles. TF in association with FVIIa has been associated with increased rates of metastasis and angiogenesis due to the complex's participation in intracellular signaling, thrombin production and fibrin deposition (12-19). Metastatic cancer cells can express as much as 1000 fold higher levels of TF than non-metastatic cancer cells (16), and TF found on the surface of breast cancer cells has been shown to be functional in binding to its partner protease, factor VIIa (20). The expression of TF can be induced in various cell types by an assortment of agents, such as lipopolysaccharide, a bacterial wall coat (21-23), serum growth factors, thrombin and fibrin (24-30). When TF is overexpressed by means of transfection, procoagulant activity is significantly increased as well as rates of tumor cell invasion and primary tumor growth (17). Since thrombin and fibrin are products of the pathway triggered by factor VIIa binding to TF, a dangerous positive feedback loop can be created upon ligation of TF. This loop is especially dangerous because thrombin not only cleaves fibrinogen, leading directly to the formation of a thrombus, but thrombin may also support the invasion of cancer cells by causing the degradation of the subendothelial matrix, which allows them to detach and metastasize to other parts of the body (31-32). Once cells have metastasized, fibrin formation becomes particularly important as it serves as a matrix for the interaction of tumor cells through their adhesive receptors. This interaction may serve to alter the expression of genes relevant for invasion of cancer cells and tumor growth (33). The deposition of fibrin plays a second role in successful tumor metastasis in that it is necessary for the arrest of cancer cells at distant sites from their source (34). Evidence for this TF:VIIa/thrombin/fibrin loop manifests itself in the observation that TF in breast cancer cells co-localizes with cross-linked fibrin (20).

Another important molecule that co-localizes with TF in breast cancer tissue is vascular endothelial growth factor (VEGF), which is secreted by these cells upon binding of factor Xa to the factor VIIa/TF complex (34, 35). VEGF promotes angiogenesis, or the growth of new blood vessels, through signaling through the VEGF receptor-2, and is one of many examples of TF acting as a signaling molecule. TF was first postulated to be a signaling molecule almost 15 years ago based on its structural similarities to the cytokine receptor superfamily (36). Since then, TF has been found to participate in diverse signaling pathways which may alter the growth, adhesive, and migratory properties of cancer cells, alter the transcription of certain genes, and initiate angiogenesis in the area immediately adjacent to the tumor (37-44). It is generally agreed that, in order for TF to act in signaling, it must be bound to active factor VIIa; however, the exact mechanism by which the signal is transduced is yet to be determined. It is possible that TF:VIIa activates a signaling receptor directly or indirectly through activation of Xa and thrombin production. In either case, regulation of the signaling events would best be achieved by blocking TF:VIIa activity so as to avoid initiation of signaling. Two independent groups have shown that VIIa is able to induce a significant p42/p44 mitogen-activated protein kinase (MAPK) signal in different cell types transfected with TF, and that phosphorylation of p42/p44 MAPK was not observed after treatment of the cells with thrombin, Xa or a protease activated receptor-1 (PAR-1) agonist peptide. This would suggest that VIIa is acting to signal directly through a PAR (39, 45). These experiments also indicated that MAPK phosphorylation may be one of the pathways responsible for TF:VIIa induced gene transcription. Other studies have shown that other MAP kinases such as p38 and JNK are also phosphorylated in response to TF:VIIa complex formation (46). Mobilization of intracellular calcium through phosphatidylinositol-specific phospholipase C has also been shown to result from TF:VIIa complex formation in various cell types such as human umbilical vein endothelial cells (HUVEC) and Madin-Darby canine kidney cells (37-39). There are serious implications of such changes in intracellular calcium levels and MAP kinase activation because these pathways are known to affect growth, differentiation, and apoptosis which may eventually lead to oncogenic transformation, tumor cell invasion and metastasis. One obvious consequence of signal transduction through TF to MAP kinases is the alteration of gene regulation. Camerer and colleagues used differential gene expression studies to study these alterations. They found that 10nM VIIa was sufficient to upregulate the expression of 24 different mRNA species, and anti-TF antibodies were sufficient to abrogate this response. The upregulated genes included those such as *c-fos*, *egr-1*, *c-Myc*, *hbEGF*, *IL-1 β* , *IL-8*, *u-PAR*, and *collagenases 1* and *3* (40). These include transcription factors, growth factors, pro-inflammatory cytokines and genes involved in cellular reorganization and migration. Naturally, upregulation of such genes may push cells into a state in which transformation to the malignant state is more probable.

In addition, ligation of TF by factor VIIa has been clearly shown to alter cell adhesion and promote the spreading and migration of cancer cells. The actin-binding protein-280 (ABP-280) associates with the cytoplasmic tail of TF and with the cytoskeleton. Upon signal transduction through TF, ABP-280 causes actin filament rearrangement. Phosphorylation of focal adhesion protein (FAK) also occurs as a result of TF-dependent actin-filament reorganization, suggesting that TF is also important in altering cell adhesion (41). Once cells have detached from the substratum, migration through the body becomes a very important issue as patients whose cancer has metastasized have very high mortality rates compared to those whose cancer has not metastasized. As previously mentioned, metastatic cancer cells often have much higher levels of TF than non-metastatic cancer cells, and it has been shown that TF may directly promote metastasis, rather than high TF levels being the result of the altered phenotype of metastatic cancer cells. When TF receptor function was abolished, cell adherence increased significantly. This was shown by using a model in which mice were injected separately with two different human cancer cell lines. By blocking TF with a monoclonal antibody, growth of pulmonary metastases was severely limited, demonstrating a real link between TF signaling and metastasis (16). Occurrence of metastases has also been shown to be reduced by using an inactivated form of VIIa that blocks signaling by competing for binding with active VIIa (12).

Finally, one last intensely studied function of the TF:VIIa complex in cancer is the promotion of angiogenesis. In order for tumors to grow beyond one to two millimeters in diameter, the growth of new capillaries from the adjacent vasculature, or angiogenesis, must be induced by the tumor itself. As

previously mentioned, VEGF is a major player in promoting angiogenesis, and it is induced by TF, through its cytoplasmic domain. It has been shown that upon inoculation of high TF- and VEGF-producing melanoma cells into immunodeficient mice, highly vascular tumors grew *in vivo*, while when the same experiment was repeated using low TF- and VEGF-producing melanoma cells, relatively avascular tumors were produced. Transfection of TF cDNA into the low TF- and VEGF-producing cells induced the elaboration of both TF and VEGF in those cells, but more importantly restored an angiogenic phenotype *in vivo* (43). The result is that there is a significant association between high TF expression levels and high microvessel density in several types of tumors, including breast tumors (47, 48). Further investigation on malignant and benign breast tissues localized TF expression to the vascular endothelial cells of the malignant phenotype but not the benign phenotype (20).

Thus, there is significant evidence that TF may be a major player in pathways that allow, not only for increased clot formation in the vicinity of tumor cells, but also for signaling leading to enhanced growth, altered adhesion properties, and increased migration of tumor cells. In addition, TF has also been shown to induce angiogenesis. Because of TF's ability to affect so many facets of cancer progression, it is imperative to understand the methods by which TF promotes tumor formation and metastasis, so that techniques to arrest these processes can be developed. Tissue factor pathway inhibitor (TFPI) is one of the physiological inhibitors of the TF:VIIa complex, but it can only inhibit the complex in the presence of factor Xa. One study argues that immobilized TFPI can support tumor cell migration by providing an attachment site for cells with exposed TF:VIIa (13). In a more recent study, however, TFPI was found to reduce incidents of metastasis, presumably by inhibiting TF:VIIa signaling (50). This study was performed with melanoma cells, thus it would be necessary to complete analogous experiments using breast cancer cells, to determine if these cells would act similarly.

A novel way that I propose to alter TF:VIIa-induced thrombosis and signaling in breast cancer is by using another natural inhibitor of the TF:VIIa complex, antithrombin (AT). AT, in combination with heparin or heparin-like compounds, can block activation of TF:VII complexes (51), and I have also recently completed experiments confirming that AT in the presence of heparin can significantly reduce TF:VIIa activity *in vitro* in a dose-dependent manner (see Figs. 1 and 2). The proposed studies will focus on investigating the role that AT plays in regulating TF/VIIa activity in blood coagulation and signaling pathways in the context of breast cancer. The possibility of altering the negative effects of TF:VIIa by using its natural inhibitors is quite novel, and to date no one has attempted to alter cell signaling through TF:VIIa using AT. While the hypothesis I will test is novel, the reagents and knowledge necessary to complete these experiments are readily available in my laboratory and in my department and university.

Hypothesis/Purpose

The novel hypothesis to be tested contains three main parts. The first part will be to determine the efficiency of AT inhibition of TF:VIIa complexes on the surface of breast cancer cells. Second, it will be determined whether addition of AT to breast cancer cells displaying TF:VIIa complexes has an effect on signaling through these complexes. Finally, a similar question will be asked by addition of AT pre-complexed with VIIa to TF-presenting breast cancer cells. To answer these questions I will first determine thermodynamic and kinetic constants for the binding and inhibition of AT on TF:VIIa complexes on breast cancer cells in culture. These constants will be relevant to initiation of the blood coagulation cascade on breast cancer cells because measurements of factor X activation will be taken. Once determined, I can compare these values to those of endothelial cells induced to express TF on their surfaces. Thermodynamic and kinetic constants for binding of AT and inhibition of TF/VIIa can also be compared with those for TFPI. The concerted effects of AT with TFPI may be compared to the previous studies. Following these studies, signaling through the TF:VIIa complex will be evaluated and compared to signaling upon addition of AT to breast cancer cells with pre-complexed TF:VIIa on their surfaces. Similar studies will be performed using AT pre-complexed with VIIa and determining the effects on signaling in breast cancer cells displaying TF on their surfaces. Finally, the long-term effects of alteration of these intracellular signaling pathways will be determined by measuring changes in gene transcription, altered cell adhesion and rates of migration of breast cancer cells in culture. If AT is found to inhibit

TF:VIIa mediated effects on cells and blood clotting, it will represent a novel mechanism with which to slow tumor cell growth and metastasis.

Objectives

1. To determine the efficiency of AT inhibition of TF:VIIa and TF:VII complexes on the surface of breast cancer cells. This task will include determining various thermodynamic and kinetic constants for binding and inhibition by AT. Normally, AT requires heparin *in vitro* to neutralize its cognate proteases, and since endothelial cells have heparin-like molecules on their surface, they will be able to enhance AT inhibition of TF:VIIa. These cells will then be used as controls against breast cancer cells to compare thermodynamic and kinetic constants of AT binding and inhibition. In order to induce TF on endothelial cell surfaces, tumor necrosis factor α (TNF α) will be used. Efficiency of TF:VIIa neutralization by AT on breast cancer cells will then be compared to that for neutralization by TFPI. AT has been shown to neutralize TF:VII complexes, preventing the initiation of blood coagulation by blocking the first step in the cascade (51). There may be, therefore, a pathway by which TFPI and AT work in concert to more efficiently prevent triggering of this cascade.

2. To determine whether addition of AT to TF:VIIa complexes on the surface of breast cancer cells can inhibit intracellular signaling through these complexes. Studies to support this hypothesis will include controls to show that addition of VIIa to TF-presenting breast cancer cells induces an intracellular signal through measure of activation of various MAP kinases and changes in intracellular calcium stores. Further, AT's effect on these signaling pathways will be measured in similar ways on breast cancer cells with pre-complexed TF:VIIa on the surface. Longer term effects of AT's alteration of TF:VIIa signaling will be studied by measuring changes in cell adhesion to solid supports and rate of migration of breast cancer cells in culture. These studies will then be compared to similar studies using TFPI in the place of AT, and TFPI and AT in concert.

3. To determine whether VIIa:AT complexes can bind to breast cancer cell surfaces and whether they have an effect on intracellular signaling. Studies to complete this task will be similar to those for objective 2, except that AT will be pre-complexed with VIIa and TF. In such a way, stable complexes of inactivated protease and protease inhibitor (VIIa:AT) are released from TF upon its formation, and will be used rather than complexing TF:VIIa with AT on the cell surfaces. It will then be determined whether these complexes are internalized by the cells, and if so, by what pathway, or with which receptor.

Methods and Experimental Design

General Methods and Materials: All of these methods and materials will be used repeatedly to achieve all or most of my objectives. It is therefore more convenient to describe them together in a separate methods section. All of the materials listed in the methods section are either already available in this laboratory, or can be readily obtained. Recombinant human factor VIIa is purchased from American Diagnostica Inc. AT is from Enzyme Research Labs. Chromozym tPA, the substrate for the chromogenic amidolytic activity assays, is from Roche. D-Phe-Pro-Arg-ANSNH-C₆H₁₁, the substrate for the fluorogenic amidolytic activity assays, is from Haematologic Technologies, Inc. Spectrozyme FXa, the chromogenic substrate for X activation assays, is from American Diagnostica, Inc. TF, factor X, and factor Xa have all been previously purified in our laboratory. TFPI is also available in our laboratory and was provided to us by George Broze at Washington University in St. Louis. There are three plate readers in the laboratory for performing amidolytic activity assays and X activation assays, and other assays, as needed. Two of the plate readers are appropriate for taking measurements of visible light and UV light, and are Molecular Devices VERSAmaxes. The other plate reader is appropriate for taking fluorescence measurements, and is a Molecular Devices SpectraMax GeminiXS. I, and my colleagues in the laboratory, have extensive experience with these reagents and instruments. **Task 1: To determine the efficiency of AT inhibition of TF:VIIa and TF:VII complexes on the surface of breast cancer cells.** Studies will be performed to determine the association rate of AT with TF:VIIa complexes on the surface of various breast cancer cells, including MCF-7, MDA-MB-231, and T47D cells, which have been shown in the past to express TF on their surfaces (52-54). Vascular endothelial cells will be used as a control in these experiments because they can be induced to express TF on their surfaces, and also, heparin-like molecules that will

serve to activate AT are also present (55,56). TF will be induced in these cells using $\text{TNF-}\alpha$. In order to determine how readily AT associates with TF:VIIa on the cell membrane, an ELISA that has been developed in our laboratory will be used. Cells will be incubated first with factor VIIa, and then with AT. The cells will be incubated at 37°C to allow complexes to form between TF:VIIa and AT. As the reaction proceeds, AT will be expelled from the membrane in the form of VIIa:AT complexes. The interaction of these proteins is calcium dependent, so after various time points, EDTA will be added to the cells to dissociate the complexes from the cells. Samples of the tissue culture medium will then be applied to an ELISA plate containing bound anti-factor VIIa antibody. These samples will be incubated to allow the antibody to capture VIIa and VIIa:AT. Several washes will be done, followed by the addition of an anti-AT antibody. These antibodies will only bind to VIIa:AT complexes, and so will give an indication of how well AT interacts with TF:VIIa on the cell surfaces. Next, an enzyme-conjugated secondary antibody will be bound to the anti-AT antibody, and a colorimetric substrate will be added. Amount of color development will be quantitated in the plate reader, and absorbance will correlate with amount of complex present in the well. An appropriate standard curve will be constructed with known amounts of the VIIa:AT complex. In short, these complexes are formed by adding an excess of AT to a known amount of factor VIIa in the presence of TF and heparin, and a chromogenic assay is used to quantitate remaining activity of factor VIIa. Once all activity has been lost, it can be assumed that there is a 1:1 ratio of factor VIIa and AT, and the concentration of the complex is equal to the concentration of the added factor VIIa. These techniques are routine in our laboratory. Finally, determining whether AT is able to inhibit cell bound TF:VIIa complexes, X activation assays will be used. This assay measures TF:VIIa activity by quantitating the initial rate of reaction of conversion of factor X to factor Xa. Briefly, TF and VIIa are allowed to react in order to form a complex, in the presence of calcium. Factor X is added to the mixture and incubated for varying time points, during which it will be converted to factor Xa. The reaction is stopped at each time point by dilution into a cold stop buffer containing detergent and EDTA to chelate calcium ions. The newly converted factor Xa is then measured in a chromogenic amidolytic activity assay using the above substrate and plate reader. By determining the rate of hydrolysis of the Xa substrate we can back-calculate to obtain the rate of conversion of factor X to factor Xa by TF:VIIa. This assay will be done on the surface of breast cancer cells and endothelial cells, and will be adapted for my hypotheses by adding AT to the original TF:VIIa mixture to determine if factor X activation is slowed in the presence of AT. By using varying time points and concentrations of AT, an inhibition rate can be determined. I have already had experience determining rate constants with AT and TF:VIIa *in vitro*, and have three years of experience culturing breast cancer cells and endothelial cells, therefore, adapting this technique to tissue culture should not present any problems. All of these experiments will then be repeated using TFPI alone or TFPI in combination with AT to further elucidate the natural TF:VIIa neutralization mechanism.

Task 2: To determine whether addition of AT to TF:VIIa complexes on the surface of breast cancer cells can inhibit intracellular signaling through these complexes. Preliminary studies will be done to attempt to confirm that factor VIIa binding to TF on the surface of cells, in this case breast cancer and endothelial cells induced with $\text{TNF}\alpha$, can induce an intracellular signal. I will test this by measuring MAP kinase activation in the cells that are treated with factor VIIa. In order to test for MAP kinase activation cells will be serum starved for 24 hours, and then treated with 10nM factor VIIa for 10 minutes. Cells will then be washed with PBS, harvested and cell extract will be subjected to separation by SDS-PAGE electrophoresis. Western blots will then be used to detect changes in MAP kinase activation with phospho-MAP kinase specific antibodies, such as those for p42/p44 MAP kinase or p38 MAP kinase. Control experiments will include cells that are serum starved for 24 hours but not treated with factor VIIa. Measurement of intracellular calcium stores is an additional method to confirm VIIa-induced cell signaling. There are several techniques for doing so, but one possible method we could use in our laboratory is one outlined by Young, Wu, and Rozengurt (57). Briefly, cells are treated with a fluorescent calcium indicator called Fura-2 for 45-60 minutes at 37°C , and then ratios of images are obtained at 1.5 second intervals. The average ratio intensity over a region in a particular cell is defined and converted to calcium ion concentration. Cells are then compared, and experiments where cells are pretreated with factor VIIa will be compared to cells that were not pretreated with factor VIIa. After these preliminary

studies have been completed I will move on to pre-treating breast cancer cells with factor VIIa followed by AT to determine how AT affects MAP kinase phosphorylation states and intracellular calcium concentrations. Alterations in gene expression after treatment with AT can be investigated by use of real time polymerase chain reaction (RT-PCR) methods on mRNAs that are known to be induced upon factor VIIa binding to TF (40). Next, the long term effects of AT on TF:VIIa signaling will be studied by determining how cell adhesion and migration change in response to treatment with AT. In order to determine if cell adhesion is altered I will adapt a protocol from Ott *et. al.* (41) in which polystyrene plates are coated with fibronectin and poly-L-lysine in Tris-buffered saline containing calcium. Wells are then washed and blocked with BSA. Next, serum starved cells are harvested and resuspended in the appropriate medium, and incubated for 30 minutes at room temperature. Factor VIIa is then added to the plate and cells are mixed and seeded onto the plates for one hour. Nonadherent cells are removed by gentle washing, and cells with a spread-out morphology are counted by phase contrast microscopy. Adhesion is then quantified using a colorimetric assay (Promega Corp., Madison, WI). Breast cancer cells can be subjected to this test because they contain TF on their surfaces. These assays can be compared with those for endothelial cells induced to express TF. To determine AT's effect on cell adhesion, AT can be added with the cells to determine if AT helps to complex the cells to the plates or if it inhibits binding. Alternatively, AT can be added with the VIIa, or cells can be pretreated with AT to determine an approximate mechanism of binding by which AT acts to alter cell adhesion to the plates. Finally, AT's effects on cell migration will be determined by using *in vitro* techniques. Binding of factor VIIa to TF has been shown to modulate chemotaxis, independent of coagulation however, AT's effects on this phenomenon have not been evaluated. Migration will be analyzed using modified Boyden chambers. The under surface of a polycarbonate membrane in a modified Boyden chamber is coated with Matrigel (Becton Dickinson Labware) diluted in Hepes-buffered tissue culture medium. The membranes are blocked with BSA and the lower compartments of the migration chamber are filled with breast cancer cells suspended in the same Hepes-buffered tissue culture medium. Migration chambers are then incubated at 37°C in 5% CO₂, and stationary cells are removed from the upper side of the membrane. Migrated cells are fixed in 3.7% paraformaldehyde in PBS and stained with crystal violet in ethanol and borate. The membranes are washed and the dye is eluted with 10% acetic acid for measurement of absorbance at 600nm. Migration on BSA is subtracted from these data (41). Cells treated with factor VIIa alone, cells treated with both factor VIIa and AT, and cells that have not been treated will be compared to determine whether AT affects cell migration *in vitro*. Studies of this nature will then be repeated using TFPI instead of AT and TFPI together with AT to determine the importance of each inhibitor on intracellular signaling, gene expression, cell adhesion, and cell migration.

Task 3: To determine whether complexes of AT:VIIa can bind to breast cancer cell surfaces and whether they have an effect on intracellular signaling. These experiments can be done simultaneously with the experiments of task 2. Experiments will be quite similar except that AT will be incubated with VIIa and TF and then applied to the cells, rather than pre-treatment with factor VIIa followed by treatment with AT. The procedure to produce known concentrations of AT:VIIa complexes is outlined in "Task 1." It is known that the low density lipoprotein receptor-related protein (LRP) is responsible for clearance of factor Xa *in vivo* (58). LRP is also responsible for internalizing thrombin-antithrombin complexes (59) by way of receptor-mediated endocytosis (60). It is then quite possible that VIIa-antithrombin complexes are also cleared by LRP. We will test this hypothesis by radioactive labeling of factor VIIa followed by its complex formation with AT. These complexes will be added to the cell culture medium alone, or in the presence of LRP antagonist receptor-associated protein (RAP) or LRP antibodies. The amount of remaining labeled complex will be measured and each group, no treatment, RAP, and LRP antibody group, will be compared to determine whether LRP is involved in internalizing these complexes. This is relevant to breast cancer in that LRP has been shown to promoted *in vitro* invasiveness of breast cancer cells (61), and is also involved in signaling through cAMP-dependent protein kinase (PKA) (62). As outlined above, I will also test whether these complexes induce signaling through MAP kinase or calcium mediated pathways.

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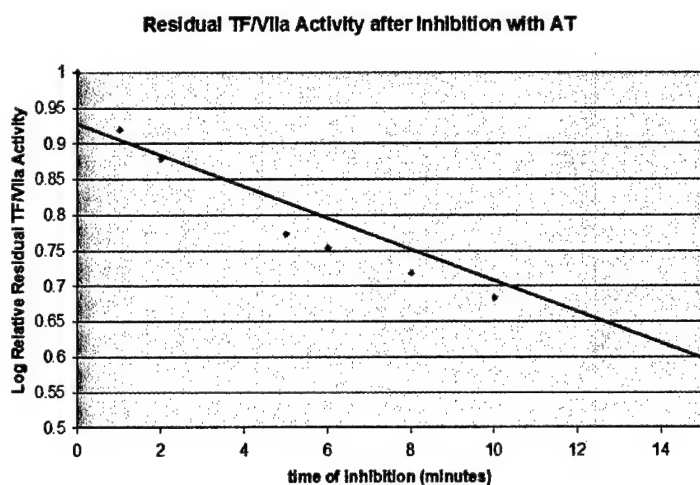


Figure 1. Physiologic levels of AT significantly inhibit TF/VIIa activity. 10nM VIIa and 100nM soluble TF (sTF) were incubated at 37°C with 5μM AT:10U/mL heparin. Reactions were stopped at by 1:10 dilution in ice cold buffer containing 20μg/mL protamine sulfate. Activity was measured at various times using a chromogenic substrate, chromozym-tPA.

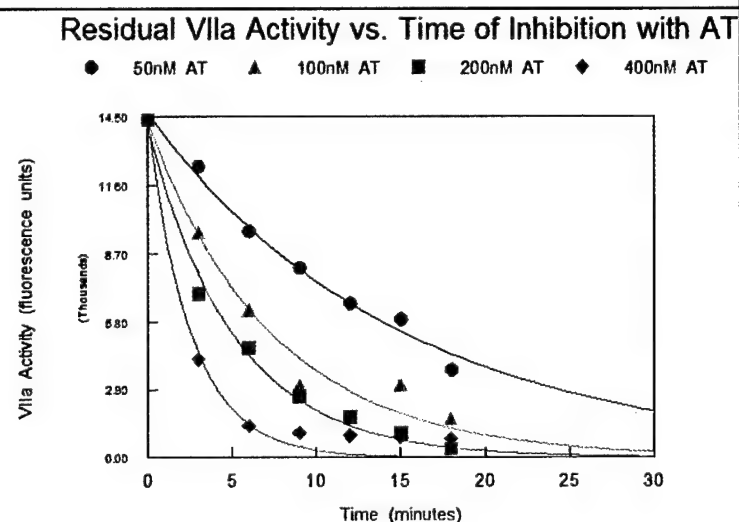


Figure 2. Rate of inhibition of TF/VIIa is AT dose-dependent. 5nM VIIa and 100nM sTF were incubated at 37°C with varying concentrations of AT:10U/mL heparin. Reactions were stopped by 1:10 dilution in ice cold stop buffer containing 100μg/mL polybrene. Residual TF/VIIa activity was measured at various times using a fluorogenic substrate, D-Phe-Pro-Arg-ANSNH-C₆H₁₁.

The Effects of Antithrombin and Tissue Factor Pathway Inhibitor on the Tissue Factor:Factor VIIa Complex on the Surface of Breast Cancer Cells: Implications on Blood Coagulation, Gene Expression, Cell Adhesion and Metastasis

Lay Abstract;

P.I. Francesca Antonaci

Thrombosis, or the abnormal clotting of blood, is the leading cause of death in breast cancer patients, and 17.6% of breast cancer patients with metastases also have underlying thromboses. The step that triggers the formation of a blood clot in the circulation occurs when a blood protein called factor VIIa interacts with a cell surface protein called tissue factor (TF). Normally, cells that are exposed to blood do not have TF on their surfaces, but many types of cancer cells, including breast cancer cells, and cells associated with breast tumors do have TF on their surfaces, and this TF is most often in contact with blood. Formation of abnormal blood clots in these patients therefore leads to such events as heart attack, deep vein thrombosis, and stroke, conditions which have very high mortality rates; in fact, metastatic breast cancer cells may express as much as 1000 fold higher levels of TF than non-metastatic breast cancer cells, which has been shown to increase rates of blood clot formation in these patients. Expression of TF on breast cancer cells not only allows for increased frequency of thromboses, but expression of TF, followed by its interaction with factor VIIa from the blood, also has many other serious consequences. TF expression has been shown to alter signaling inside of the cells, the expression of certain genes involved in growth, gene transcription, pro-inflammatory molecules, cellular reorganization and migration, and angiogenesis, or the formation of new blood vessels, a process vital for the life of a tumor. TF also directly associates with actin binding protein 280 (ABP-280) within a cell and, upon interaction with factor VIIa, allows changes to occur in the cell structure and reorganization which leads to decreased cell adhesion and increased cell migration. All of these factors taken together show that TF is very important in breast cancer pathology because it affects so many facets of the life of a cancer cell, such as growth, angiogenesis, transformation of normal cells into cancer cells, cell adhesion, and metastasis. It is therefore imperative to understand the methods by which TF promotes tumor formation and metastasis, so that techniques to arrest these processes can be developed. Tissue factor pathway inhibitor (TFPI) and antithrombin (AT) are two of the natural inhibitors of the TF:VIIa complex, and the goal of this work is to investigate their role in blood clotting, alterations in gene expression, cell adhesion, and metastasis in breast cancer.

The novel hypothesis I propose to test is of three main parts. First, it will be determined whether AT and TFPI bind to and efficiently inhibit TF:VIIa complexes on the surfaces of breast cancer cells. If so, this may be a novel mechanism by which breast cancer growth and metastasis may be slowed. Second, AT's and TFPI's effects on intracellular signaling via the TF:VIIa receptor will be studied. Longer-term effects on cell adhesion and migration will also be studied. Finally, it will be tested how VIIa:AT complexes affect these same cell properties. These complexes are formed after AT interacts with TF:VIIa complexes on cell surfaces and may also serve to promote tumor cell growth and metastasis.

While the hypothesis I propose to test is novel, all of the equipment, reagents, and knowledge I need to complete this work is already present or readily available in my laboratory.

The Effects of Antithrombin and Tissue Factor Pathway Inhibitor on the Tissue Factor:Factor VIIa Complex on the Surface of Breast Cancer Cells: Implications on Blood Coagulation, Gene Expression, Cell Adhesion and Metastasis
Technical Abstract;
P.I. Francesca Antonaci

Background: Thrombosis, or the abnormal clotting of the blood, is the leading cause of death in breast cancer patients, and 17.6% of breast cancer patients with metastases also have underlying thromboses. The step that triggers the formation of a thrombus occurs when factor VIIa from circulating blood encounters tissue factor (TF) on the surface of a cell. Normally, cells that are exposed to blood do not express TF, but many types of cancer cells, including breast cancer cells, and tumor-associated macrophages and endothelial cells do express TF. Thrombus formation in these patients leads to such events as myocardial infarction, deep vein thrombosis, and stroke, pathological conditions which have high mortality rates; in fact, metastatic breast cancer cells may express as much as 1000 fold higher levels of TF than non-metastatic breast cancer cells, which has been shown to increase thrombogenic activity. Expression of TF on breast cancer cells not only allows for increased frequency of thromboses, but expression of TF, followed by its ligation with factor VIIa also has many other serious consequences. TF expression has been shown to alter intracellular signaling through mitogen-activated protein kinases (MAPKs) and through phospholipase C-induced intracellular calcium mobilization. TF expression and ligation has also been shown to induce the transcription of 24 different genes, among which are VEGF, a potent pro-angiogenesis growth factor, transcription factors, pro-inflammatory cytokines, genes involved in cellular reorganization and migration, and other growth factors. TF's cytoplasmic domain directly associates with actin binding protein 280 (ABP-280) which allows it to directly affect cell structure reorganization, cell adhesion, and cell migration. All of these factors taken together show that TF is a very important molecule in that it influences blood clotting, cell growth, angiogenesis, oncogenic transformation, cell adhesion, and metastasis, as well as other possible cell properties, such as cell differentiation and apoptosis. It is, therefore, imperative to understand the methods by which TF promotes tumor formation and metastasis, so that techniques to arrest these processes can be developed. Tissue factor pathway inhibitor (TFPI) and antithrombin (AT) are two of the natural inhibitors of the TF:VIIa complex, and the goal of this work is to investigate their role in blocking or slowing blood clotting, alteration of gene expression, cell adhesion, and metastasis in breast cancer.

Objective/Hypothesis: The novel hypothesis to be tested contains three main parts. First, it will be determined whether AT and TFPI bind to and efficiently inhibit TF:VIIa complexes on the surfaces of breast cancer cells. Second, I will study the effects of AT and TFPI on intracellular signaling through the TF:VIIa receptor. Finally, I will determine the effects of pre-formed VIIa:AT complexes on intracellular signaling, and whether these complexes are cleared by breast cancer cells. If AT is found to inhibit TF:VIIa mediated effects on cells and blood clotting, it will represent a novel mechanism with which to slow tumor cell growth and metastasis.

Specific Aims: 1. To determine the efficiency of AT inhibition of TF:VIIa and TF:VII complexes on the surface of breast cancer cells. 2. To determine whether addition of AT to TF:VIIa complexes on the surface of breast cancer cells can inhibit intracellular signaling through these complexes. 3. To determine whether VIIa:AT complexes can bind to breast cancer cell surfaces and whether they have an effect on intracellular signaling.

Study Design: In order to achieve these goals, thermodynamic and kinetic measurements will be taken on the surface of breast cancer cells to determine how well AT and TFPI can bind to and inhibit TF:VIIa complexes. Signaling changes through MAPK and calcium flux will be determined with and without TF:VIIa inhibitors, while long-term changes caused by AT and TFPI, such as alterations in gene transcription, cell adhesion, and cell migration, will be evaluated. These studies will also be performed in the presence of pre-formed VIIa:AT complexes, and it will be determined how VIIa:AT complexes are cleared by breast cancer cells.

Training Plan: General course work in biochemistry, methods, and seminars have already been completed. I have already passed my preliminary examination and my cumulative examinations. Last summer I attended the Endocrine Society's meeting in San Francisco and the Serpin Structure and Function meeting in Chicago. The latter meeting was extremely relevant to the work presented in this proposal because many of the talks were on antithrombin and similar inhibitory systems. This summer I will be attending a meeting in Birmingham England on Haemostasis and Thrombosis, which is obviously relevant to the work in my new laboratory. While my new advisor, Jim Morrissey, is not known specifically as a breast cancer researcher, he is a well-known and respected scientist, especially for his vast amount of work with tissue factor. Our laboratory also collaborates with Dr. Bradford Schwartz and Dr. Naveen Manchanda, both of which work regularly with breast cancer systems. Both are M.D.'s and work closely with us. In order to continue my training I will also complete electives relevant to this proposal, such as a cell signaling course that is offered here in the spring, and I will continue to attend weekly seminars hosted by the Biochemistry Department. In the future I will also teach courses in the field of Biochemistry, and have already completed one year of teaching for the Department of Chemistry here at UIUC. Finally, if allowed to continue work with this fellowship, I will also attend the CDMRP's meeting. Please note that during the tenure of this fellowship I will only be enrolled as a graduate student, and not as a medical student, even though I am technically considered an M.D./Ph.D. student.

The Effects of Antithrombin and Tissue Factor Pathway Inhibitor on the Tissue Factor:Factor VIIa Complex on the Surface of Breast Cancer Cells: Implications on Blood Coagulation, Gene Expression, Cell Adhesion and Metastasis

Statement of Work;

P.I. Francesca Antonaci

Task 1: To determine the efficiency of AT inhibition of TF:VIIa and TF:VII complexes on the surface of breast cancer cells. (Months 1-16)

- a. Determine association rates of AT and TFPI with TF:VIIa complexes on breast cancer cell surfaces; T47D, MCF-7, MB-MDA-231 cells. Compare with rates on endothelial cell control surfaces (Months 1-10);
- b. Determine rate of inhibition of TF:VIIa complexes on breast cancer cell surfaces by AT and TFPI alone and AT with TFPI; compare with endothelial cell controls and *in vitro* inhibition rates; use factor X activation as a model system, (effects on blood coagulation) (Months 8-16);

Task 2: To determine whether addition of AT to TF:VIIa complexes on the surface of breast cancer cells can inhibit intracellular signaling through these complexes. (Months 16-30)

- a. Confirm that addition of factor VIIa to breast cancer cells and endothelial cell controls induces an intracellular signal through MAP kinases and calcium mobilization (Months 16-18);
- b. Determine effects on MAP kinase signaling and calcium mobilization in breast cancer cells upon addition of AT, TFPI, or AT/TFPI in combination (Months 18-22);
- c. Determine effects of AT, TFPI, and AT/TFPI in combination on gene expression in breast cancer cells (Months 22-26);
- d. Examine effects of AT, TFPI, AT/TFPI on breast cancer cell adhesion (Months 26-30);
- e. Study effects of AT, TFPI, AT/TFPI on breast cancer cell migration in culture (Months 26-30);

Task 3: To determine whether VIIa:AT complexes can bind to breast cancer cell surfaces and whether they have an effect on intracellular signaling. (Months 18-36)

- a. Determine effects of MAP kinase signaling and calcium mobilization upon addition of VIIa:AT complexes to breast cancer cells (Months 18-22);
- b. Determine effects of VIIa:AT complexes on gene expression in breast cancer cells (Months 22-26);
- c. Examine effects of VIIa:AT complexes on breast cancer cell adhesion (Months 26-30);
- d. Study effects of VIIa:AT complexes on breast cancer cell migration in culture (Months 26-30);
- e. Determine whether VIIa:AT complexes are cleared from breast cancer cell culture medium (Months 30-31);
- f. Determine whether VIIa:AT complexes are internalized by cell-mediated endocytosis through the LRP receptor (Months 31-36).

Determining the Nature of Inhibition of Factor VIIa by Antithrombin:

A Possible Novel Mechanism for Maintaining Hemostasis

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BACKGROUND

Factor VIIa (VIIa) is a serine protease that normally circulates in the bloodstream at very low concentrations (<100pM). When a blood vessel incurs an injury, tissue factor (TF), a glycoprotein expressed on certain cells outside of the vasculature, is exposed to VIIa circulating in the blood, thus initiating the clotting cascade. Together, TF and VIIa form a potent enzyme capable of maintaining hemostasis, but the complex is also involved in pathogenic thrombosis. Low levels of TF have been found circulating in normal blood, and, in theory, when VIIa comes into contact with these forms of TF, formation of a thrombus is possible. This thrombus could potentially cause myocardial infarction, stroke, or other pathological conditions that may eventually lead to death. Under normal conditions, however, fibrinolysis is not formed in the bloodstream, and our hypothesis is that VIIa circulates in an inhibited form.

Antithrombin (AT) is a member of the serpin family of protease inhibitors, and is capable of inhibiting a large spectrum of blood proteases. AT is able to inhibit VIIa, in the presence of TF and heparin, so we propose that AT also plays a role in inhibiting VIIa through a competitive mechanism, and that this is the method by which hemostasis is normally maintained, even in the absence of circulating TF.

METHODS

Anticlotting activity assays: AT (Enzyme Research Labs) was incubated with VIIa (American Diagnostica Inc.) and heparin (Sigma) in heparin buffered saline with 5mM CaCl₂ for 20 minutes at 37°C. Residual activity was measured using a small substrate, chromozym t-PA (Roche), which produces yellow color upon cleavage by the enzyme. Increase in color is measured by monitoring for 20 minutes at 405nm and 57°C. All experiments were compared to control experiments lacking AT.

To measure recovery of VIIa activity after inhibition by AT, the above reaction is diluted ten fold into heparin buffered saline with 5mM CaCl₂. The reaction mixture is then further incubated for 20 minutes at 37°C in order to allow dissociation, and residual activity is measured as above.

Clotting assays: In order to determine whether dilution of VIIa:AT reactions can lead to full recovery of activity (covalent complex), clotting assays were performed. 0.4mM AT was incubated with 20mM VIIa and 10U/ml heparin in heparin buffered saline with 5mM CaCl₂ for 20 minutes at 37°C, and then diluted 100 fold into the same buffer. The reaction was reconstituted for 20 minutes at 37°C to allow for dissociation, and then tested in a clotting assay. In short, 50µl of each reaction mixture, with 0.1mM added, reconstituted TF (prepared by Stephanie Smith), was incubated for 2 minutes at 37°C with 17mM CaCl₂, followed by addition of 50µl of VII deficient plasma (George King Bio-medical Inc.). The prothrombin time was measured using a Diagnostica Sego 574 coagulometer. Control experiments were performed in parallel without AT. Standard curves were constructed using known amounts of VIIa.

Western blotting: To visualize VIIa:AT complexes, varying amounts of VIIa were incubated with different combinations of soluble TF (prepared by Guyon Li), heparin, and AT for 20 minutes at 37°C. Reactions were boiled for 5 minutes in SDS non-reducing sample buffer, and loaded onto a 12% SDS-PAGE gel. Bands were separated, and blotted onto a nitrocellulose membrane. Membranes were probed with a polyclonal antibody against AT (DAKO).

Kinetic measurements: To determine whether the inhibition of VIIa by AT is competitive, antidiolitic activity assays were performed using varying amounts of chromozym t-PA and AT with 50mM VIIa. Data were plotted using a two-site ligand fit (Michaelis-Menten equation fit), and using the Lineweaver-Burke method.

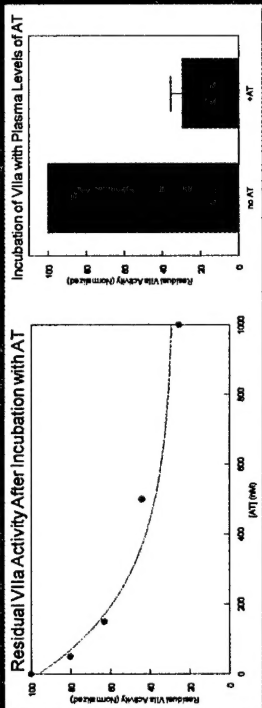


Figure 1. AT inhibits VIIa in the absence of TF. (Left Panel) A 12-fold excess of AT over VIIa (75nM) is capable of 70% inhibition at 20% the plasma level of AT. (Right Panel) Plasma levels of AT (5nM) inhibit 30nM VIIa by 70% after 20 minutes at 37°C. Plasma levels of VIIa are <100pM, indicating that AT may be a very efficient inhibitor of plasma VIIa.



Figure 2. AT forms very little covalent complex with VIIa in the absence of TF. VIIa was incubated with and without AT and TF for 20 min at 37°C. Samples were boiled in non-reducing SDS buffer and blotted with an anti-AT antibody.

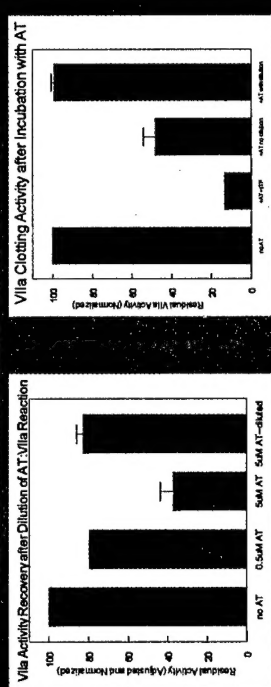


Figure 3. VIIa activity is recovered upon dilution of VIIa:AT reactions. AT (5nM) and 60nM VIIa were incubated with 10 fold dilution of heparin for 20 min at 37°C, followed by 10 fold dilution and reconstituted. Final antidiolitic activity was measured and multiplied by the dilution factor to adjust. (Note that 10-fold diluted 5nM AT still contains 0.5nM AT).

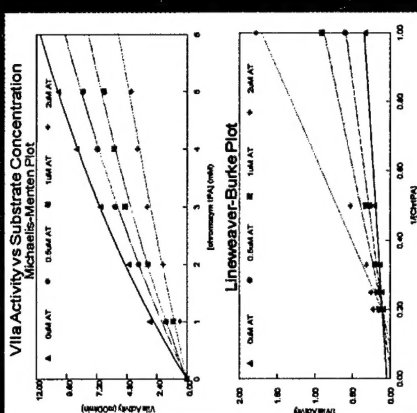


Figure 4. VIIa activity is completely recovered in clot assays after dilution of VIIa:AT reactions. 20nM VIIa was incubated with 0.4mM AT and 10U/ml heparin for 20 min at 37°C. Reactions were diluted 1000 fold and residual activity was measured as the prothrombin clotting time.

CONCLUSIONS

- AT, in the presence of heparin, is able to inhibit VIIa:TF with a rate constant of $1.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (data not shown).
- AT, in the presence of heparin, can inhibit VIIa alone by as much as 70% after 20 minutes at 37°C.
- Very little VIIa:AT complex is formed in the absence of TF, suggesting a novel mechanism for inhibition that may be reversible: in the presence of TF, AT and VIIa normally form an SDS-stable covalent complex.
- Antidiolitic activity assays and clotting assays indicate that full recovery of VIIa activity can be obtained, by dilution, after incubation of AT and VIIa in the absence of TF, showing that the mechanism is competitive inhibition (further studies in progress).

FUTURE DIRECTIONS

- Reconfirm these data and complete studies on Michaelis-Menten kinetics.
- Determine applicability of this model in a tissue culture system.
- Repeat these studies in the absence of heparin and in the presence of other glycosaminoglycans.
- Examine whether TF cells with glycosaminoglycans present on their surface can support formation of VIIa:AT complexes.
- Determine whether AT and VIIa can form complexes in normal plasma, with and without heparin.
- Study the effects of VIIa:AT complexes on intracellular signaling.

We wish to thank Stephanie Smith and Guyon Li for their preparations of reconstituted and soluble TF. Thanks to Collin Waters and Shih-hon Li for help in preparation of this poster. Special thanks to the NIH/NIH (NIH) and the U.S. Army Medical Research and Materiel Command Breast Cancer Research Program for funding.